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INVENTORS: JEROME S. HARMS
ROBERT D. BREMEL
KURT A. EAKLE
GARY A. SPLITTER

TITLE: INDUCIBLE PROTEIN EXPRESSION SYSTEM

ATTORNEYS: FRANK C. NICHOLAS
CARDINAL LAW GROUP
1603 ORRINGTON AVENUE
SUITE 2000
EVANSTON, IL 60201
(847) 905-7111

INDUCIBLE PROTEIN EXPRESSION SYSTEM

RELATED APPLICATION

This application claims priority to U. S. Provisional Patent Application No. 60/442,103, filed on January 23, 2003 and titled "Tax Inducible Expression System" by Jerome S. Harms, et al., its entire disclosure is incorporated by reference.

GOVERNMENT SUPPORT

This invention was made partially with government support under Grant No CA88752-03 awarded by the National Institute of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to gene expression. In particular, the present invention relates to inducible gene expression systems and a method thereof.

BACKGROUND OF THE INVENTION

The advent of recombinant DNA technology has made it possible to produce foreign proteins in mammalian cells through the introduction of foreign DNA encoding such proteins. Mammalian expression systems are available in which the foreign protein is constitutively expressed from an active promoter. This results in the continual expression of the foreign gene. Viral promoters are commonly used as regulatory elements in gene therapy vectors due to their strong activity in various cell lines *in vitro*. A widely used promoter in expression systems is the human cytomegalovirus immediate-early gene (CMV) promoter. The CMV promoter induces high-level constitutive expression in a variety of cell lines (Fitzsimons *et al.*, Methods. 28:227 [2002]).

Frequently, the abundant presence of the foreign protein is toxic to the host cell. As a result of constitutive expression, the host cell population may become moribund and perish. Furthermore, the abundant presence of the toxic protein exerts selective pressures on the host cells which can result in the emergence of a cell population containing mutated versions of the foreign DNA, which express grossly modified protein or which have deleted the foreign gene. As a result, commercially useful levels of constitutive expression may never be maintained in the recombinant cell population.

Efforts to combat this shortcoming have resulted in the development of inducible mammalian expression systems that control the expression of the foreign protein. Inducible expression can be achieved by using promoters that are controlled by the presence or absence of a specific regulator. Another means of controlling foreign gene expression involves the use of a promoter that becomes more active in the presence of a specific activator protein. A foreign gene under the control of such a promoter is expressed at high levels only following the induction of synthesis of the activator. However, many of these inducible systems currently available suffer from decreased levels of expression and "leaky" control of expression (i.e., unwanted, low-level protein expression). A more ideal inducible system would have 1) low basal expression levels; 2) high induced expression; and 3) inducer-specific, modulated expression (Xu *et al.*, Gene. 309:145 [2003]).

Accordingly, it would be desirable to provide a tightly regulated and highly inducible protein expression system that would overcome the aforementioned and other disadvantages.

SUMMARY OF THE INVENTION

A first aspect of the invention provides an inducible gene expression system. The system includes a first inducible gene expression system including a first vector comprising at least one retroviral promoter and at least one factor to induce the retroviral promoter. At least one gene product is expressed in proportion to retroviral promoter induction.

A second aspect of the invention provides a method of gene expression. The method includes providing a first vector comprising at least one retroviral promoter and providing at least one factor corresponding to the retroviral promoter. The retroviral promoter is induced with the at least one factor. At least one protein is expressed based on the induction of the retroviral promoter.

A third aspect of the invention provides an inducible gene expression system. The system includes first vector means comprising at least one retroviral promoter. Means for inducing the retroviral promoter are provided. Further included are means for expressing at least one protein based on the induction of the retroviral promoter.

The foregoing and other features and advantages of the invention will become further apparent from the following detailed description of the presently preferred embodiments, read in conjunction with the accompanying drawings. The detailed description and drawings are merely illustrative of the invention, rather than limiting the scope of the invention being defined by the appended claims and equivalents thereof.

DESCRIPTION OF THE FIGURES

FIG. 1 shows the FACS data comparing expression constructs used in some embodiments of the present invention;

FIG. 2 shows the nucleic acid sequence of the BLV promoter (SEQ ID NO:1);

FIG. 3 shows the nucleic acid (SEQ ID NO:2) and amino acid (SEQ ID NO:7) sequence of BLV Tax;

FIG. 4 shows the nucleic acid sequence of the HTLV promoter (SEQ ID NO:4);

FIG. 5 shows the nucleic acid (SEQ ID NO:3) and the amino acid (SEQ ID NO:8) of HTLV Tax;

FIG. 6 shows the nucleic acid sequence of the HIV promoter (SEQ ID NO:5);

FIG. 7 shows the nucleic acid (SEQ ID NO:6) and amino acid (SEQ ID NO:9) of HIV Tat;

FIG. 8 shows a map of pLBC-BTax;

FIG. 9 shows a map of pLBC-BTaxW;

FIG. 10 shows the nucleic acid sequence of pLBC-BTaxW (SEQ ID NO:10);

FIG. 11 shows a map of pLNBLV-M4W;

FIG. 12 shows the nucleic acid sequence of pLNBLV-M4W (SEQ ID NO:11);

FIG. 13 shows a map of pLNBlv-YFP;

FIG. 14 shows the nucleic acid sequence of pLNBlv-YFP (SEQ ID NO:12);

FIG. 15 shows a map of pLNHiv-YFP;

FIG. 16 shows the nucleic acid sequence of pLNHiv-YFP (SEQ ID NO: 13);

FIG. 17 shows a schematic representation of BLV promoter used in comparison studies. The BLV promoter (BLVp) consisting of the U3 region of the 5'LTR of BLV includes the basic elements of transcription start site (+1), CAAT (nt -97/-92) and TATA (nt –43/-37) boxes as shown. Unique to the BLVp are the three imperfectly conserved 21bp sequences known as the Tax Responsive Elements (TxRE). The numbers following the TxRE designation represent its position relative to the transcription start site. Each TxRE contains a consensus E box-binding motif overlapping an imperfect cyclic AMP responsive element motif (CRE/Ebox). Additionally, the BLVp contains a glucocorticoid responsive element (GRE), Nuclear Factor Kappa Binding motif (NFkB), and B cell specific PU.1 or Spi-B transactivator binding motif (PU.1/Spi-B). The transcription elements are not drawn to scale;

FIGS. 18A and 18B show BLVp and CMVp activity comparison in D17, FLK, primary cow B cells, and BL3.1. Relative light units (RLU) of luciferase activity driven by either the BLV promoter (BLVp) or CMV promoter (CMVp) of 1 x 10^6 stably transduced cells was measured during a 10 s period. Bars represent the arithmetic mean and variance of 10 experiments. *P<0.05; **P<0.001 determined by T-test;

FIG. 19 shows that BLV infection enhances BLVp activity but has no effect on CMVp activity. D17 cells or D17 cells infected with and productively expressing BLV (D17+BLV) were transduced with luciferase expression vectors. Relative light units (RLU) of luciferase activity driven by either the BLV promoter (BLVp) or CMV promoter (CMVp) of 1 x 10^6 stably transduced cells was measured during a 10 s period. Bars represent the arithmetic mean and variance of 10 experiments. **P<0.001 determined by T-test; and

FIGS. 20A and 20B show that BLV Tax expression significantly enhances BLVp activity but has no effect on CMVp activity. D17 cells and primary bovine B cells (D17; B cells), or D17 cells and primary bovine B cells stably transduced with a BLV Tax expression vector (D17+TAX; B cells+TAX), were assayed. Relative light units (RLU) of luciferase activity driven by either the BLV promoter (BLVp) or CMV promoter (CMVp) of 1 x 10^6 stably transduced cells were measured during a 10 s period. Bars represent the arithmetic mean and variance of 10 experiments. **P<0.001 determined by T-test.

DETAILED DESCRIPTION OF THE INVENTION

In some embodiments, the present invention provides eukaryotic gene expression vectors, systems, and methods based on inducible retroviral promoters. For example, in some embodiments, the present invention provides Tax inducible expression vectors utilizing the bovine leukemia virus (BLV) promoter. The BLV, a C-type retrovirus, is the cause of enzootic bovine leukemia (Miller and Olson. 1987. p. 87-90. *In* A. Burny and M. Mammerickx (eds). Enzootic Bovine Leukosis and Bovine Leukemia Virus. Martinus Nijhoff Publishing, Boston). The BLV promoter alone does not typically produce very high levels of gene expression. However, the BLV promoter is transactivated by Tax (Kiermer *et al.*, J. Virol. 72:5526 [1998]), a transcriptional activator of the BLV long terminal repeat that influences the expression of many BLV genes (Jeang *et al.*, J. Virol. 71:6277 [1997]).

The vectors of the present invention may provide several advantages over currently available expression systems. The inducible vectors of the present invention may provide tight on/off regulation of expression. Unlike many currently available systems, background, or leaky, expression of the gene in the absence of the inducer is extremely low. The promoters of the present invention, however, may offer higher inducible levels and faster response times. The vectors of the present invention may further offer high absolute expression levels. Maximum expression levels may be higher than expression levels obtained from constitutive promoters such as the CMV promoter. One particular advantage of the present design relates to the utilization of transcriptional activation, rather than repression to control expression.

I. Expression Vectors

In some embodiments, the present invention may provide retroviral expression vectors comprising retroviral promoters including an inducible element, a gene of interest, as well as other components necessary for expression of the gene of interest. In some embodiments, the inducer is provided separately from the gene of interest. As used herein, the term "vector" refers to any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, retrovirus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. Further, the term "nucleotide sequence of interest" refers to any nucleotide sequence (*e.g.*, RNA or DNA), the manipulation of which may be deemed desirable for any reason (*e.g.*, treat disease, confer improved qualities, *etc.*), by one of ordinary skill in the art. Such nucleotide sequences include, but are not limited to, coding sequences, or portions thereof, of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and non-coding regulatory

sequences that do not encode an mRNA or protein product (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*).

A. Promoters

In some embodiments, the present invention provides expression vectors comprising a retroviral vector comprising an inducible element. In some embodiments, the retroviral vector promoter is a Bovine leukemia virus (BLV) promoter (SEQ ID NO:1). The BLV promoter may include an inducer-responsive element (*e.g.*, a Tax responsive element). The present invention, however, is not limited to the BLV promoter. Other suitable inducible retroviral promoters are contemplated including, but not limited to, the human immunodeficiency viruses (HIV-1 and HIV-2) promoters, feline immunodeficiency virus (FIV) promoter, simian immunodeficiency virus (SIV) promoter, caprine virus, human foamy virus and the human T-lymphocyte leukemia viruses (HTLV-1, HTLV-2, and HTLV-3) promoters.

B. RNA Export Elements

In other embodiments, the vectors may be modified by incorporating an RNA export element (*See, e.g.*, U.S. Pat. Nos. 5,914,267; 6,136,597; and 5,686,120; and WO99/14310) either 3' or 5' to the nucleic acid sequence encoding the protein of interest. It is contemplated that the use of RNA export elements may allow high levels of expression of the protein of interest without incorporating splice signals or introns in the nucleic acid sequence encoding the protein of interest.

In certain embodiments, the vector additionally may include a RNA transport signal from woodchuck hepatitis virus response element (WPRE). The woodchuck hepatitis virus post transcriptional enhancer may enhance the cytoplasmic levels of RNA as well as the translation of the target protein. Experiments have demonstrated that inclusion of the WPRE can increase the expression of a reporter gene expression under the control of the BLV promoter activated by BLV Tax.

C. Vectors

The present invention is not limited to any particular vector. Indeed, the use of a variety of vectors is contemplated, including, but not limited to plasmids, cosmids, bacterial artificial chromosomes, yeast artificial chromosomes, adeno-associated virus vectors, and adenovirus vectors. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Such vectors include, but are not limited to, the following vectors: 1) Bacterial -- pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); and 2) Eukaryotic -- pWLNEO, pSV2CAT, pOG44, PXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). Any other plasmid or vector may be used as long as they are replicable and viable in the host. In some embodiments of the present invention, mammalian expression vectors comprise an origin of replication, a suitable promoter and enhancer (*e.g.*, BLV and Tax), and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. In other embodiments, DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

In some embodiments of the present invention, recombinant expression vectors include origins of replication and selectable markers permitting transformation of the host cell (*e.g.*, dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or tetracycline or ampicillin resistance in *E. coli*). As used herein, the term "host cell" refers to any eukaryotic cell (*e.g.*, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located *in vitro* or *in vivo* (*e.g.*, in a transgenic organism). Further, the term "selectable marker" refers to a gene that encodes an enzymatic activity that confers the ability to grow in medium lacking what would otherwise be an essential nutrient (*e.g.*, the *HIS3* gene in yeast cells); in addition, a selectable marker may confer resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed.

In other embodiments, the expression vector may also contain a ribosome binding site for translation initiation and a transcription terminator. In still other embodiments, the vector may include appropriate sequences for amplifying expression.

In some embodiments, the vector is a retroviral vector. Retroviruses (i.e., family Retroviridae) are divided into three groups: the spumaviruses (*e.g.*, human foamy virus); the lentiviruses (*e.g.*, human immunodeficiency virus and sheep visna virus) and the oncoviruses (*e.g.*, MLV, Rous sarcoma virus).

Retroviruses are enveloped (*i.e.*, surrounded by a host cell-derived lipid bilayer membrane) single-stranded RNA viruses, which infect animal cells. When a retrovirus infects a cell, its RNA genome is converted into a double-stranded linear DNA form (*i.e.*, it is reverse transcribed). The DNA form of the virus is then integrated into the host cell genome as a provirus. The provirus serves as a template for the production of additional viral genomes and viral mRNAs. Mature viral particles containing two copies of genomic RNA bud from the surface of the infected cell. The viral particle comprises the genomic RNA, reverse transcriptase and other *pol* gene products inside the viral capsid (which

contains the viral *gag* gene products), which is surrounded by a lipid bilayer membrane derived from the host cell containing the viral envelope glycoproteins (also referred to as membrane-associated proteins).

The organization of the genomes of numerous retroviruses is well known to the art and this has allowed the adaptation of the retroviral genome to produce retroviral vectors. The production of a recombinant retroviral vector carrying a gene of interest is typically achieved in two stages.

First, the gene of interest is inserted into a retroviral vector that contains: the sequences necessary for the efficient expression of the gene of interest (including promoter and/or enhancer elements which may be provided by the viral long terminal repeats (LTRs) or by an internal promoter/enhancer and relevant splicing signals); sequences required for the efficient packaging of the viral RNA into infectious virions (*e.g.*, the packaging signal (Psi), the tRNA primer binding site (-PBS) the 3' regulatory sequences required for reverse transcription (+PBS)); and the viral LTRs. The LTRs contain sequences required for the association of viral genomic RNA, reverse transcriptase and integrase functions, and sequences involved in directing the expression of the genomic RNA to be packaged in viral particles. For safety reasons, many recombinant retroviral vectors lack functional copies of the genes that are essential for viral replication (these essential genes are either deleted or disabled); therefore, the resulting virus is said to be replication defective.

Second, following the construction of the recombinant vector, the vector DNA may be introduced into a packaging cell line. Packaging cell lines provide proteins required in *trans* for the packaging of the viral genomic RNA into viral particles having the desired host range (*i.e.*, the viral-encoded gag, pol and env proteins). The host range is controlled, in part, by the type of envelope gene product expressed on the surface of the viral particle. Packaging cell lines may express ecotrophic, amphotropic or xenotropic envelope gene products. Alternatively, the packaging cell line may lack sequences encoding a viral

envelope (env) protein. In this case the packaging cell line will package the viral genome into particles that lack a membrane-associated protein (*e.g.*, an env protein). To produce viral particles containing a membrane associated protein that will permit entry of the virus into a cell, the packaging cell line containing the retroviral sequences may be transfected with sequences encoding a membrane-associated protein (*e.g.*, the G protein of vesicular stomatitis virus (VSV)). The transfected packaging cell may then produce viral particles that contain the membrane-associated protein expressed by the transfected packaging cell line. These viral particles, which contain viral genomic RNA derived from one virus encapsulated by the envelope proteins of another virus, are said to be pseudotyped virus particles.

The retroviral vectors of the present invention may further be modified to include additional regulatory sequences (*e.g.*, inducible promoters of the present invention). In other embodiments, where secretion of the protein of interest is desired, the vectors may be modified by including a signal peptide sequence in operable association with the protein of interest. The sequences of several suitable signal peptides are known to those in the art, including, but not limited to, those derived from tissue plasminogen activator, human growth hormone, lactoferrin, alpha-casein, and alpha-lactalbumin.

In still other embodiments, the vector may further comprise at least one internal ribosome entry site (IRES) sequence. The sequences of several suitable IRES's are available, including, but not limited to, those derived from foot and mouth disease virus (FDV), encephalomyocarditis virus, and poliovirus. The IRES sequence can be interposed between two transcriptional units (*e.g.*, nucleic acids encoding different proteins of interest or subunits of a multisubunit protein such as an antibody) to form a polycistronic sequence so that the two transcriptional units are transcribed from the same promoter.

The retroviral vectors of the present invention may also further comprise a selectable marker allowing selection of transformed cells. A number of selectable markers may be implemented in the present invention, including, but not limited to: the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as the *neo* gene) that confers resistance to the drug G418 in mammalian cells; the bacterial hygromycin G phosphotransferase (*hyg*) gene that confers resistance to the antibiotic hygromycin; and the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the *gpt* gene) that confers the ability to grow in the presence of mycophenolic acid. In some embodiments, the selectable marker gene may be provided as part of polycistronic sequence that also encodes the protein of interest.

Viral vectors, including recombinant retroviral vectors, may provide a more efficient means of transferring genes into cells as compared to other techniques such as calcium phosphate-DNA co-precipitation or DEAE-dextran-mediated transfection, electroporation or microinjection of nucleic acids. It is believed that the efficiency of viral transfer is due in part to the fact that the transfer of nucleic acid is a receptor-mediated process (*i.e.*, the virus binds to a specific receptor protein on the surface of the cell to be infected). In addition, the virally transferred nucleic acid once inside a cell integrates in controlled manner in contrast to the integration of nucleic acids which are not virally transferred; nucleic acids transferred by other means such as calcium phosphate-DNA co-precipitation may be subject to rearrangement and degradation.

The most commonly used recombinant retroviral vectors are typically derived from the amphotropic Moloney murine leukemia virus (MoMLV) (*See e.g.*, Miller and Baltimore Mol. Cell. Biol. 6:2895 [1986]). The MoMLV system has several advantages: 1) this specific retrovirus can infect many different cell types, 2) the established packaging cell lines are available for the production of recombinant MoMLV viral particles and 3) the transferred genes are permanently integrated into the target cell chromosome. The established MoMLV vector

systems comprise a DNA vector containing a small portion of the retroviral sequence (*e.g.*, the viral long terminal repeat or "LTR" and the packaging or "psi" signal) and a packaging cell line. The gene to be transferred is inserted into the DNA vector. The viral sequences present on the DNA vector provide the signals necessary for the insertion or packaging of the vector RNA into the viral particle and for the expression of the inserted gene. The packaging cell line provides the proteins required for particle assembly (Markowitz *et al.*, J. Virol. 62:1120 [1988]).

The low titer and inefficient infection of certain cell types by MoMLV-based vectors has been overcome by the use of pseudotyped retroviral vectors that contain the G protein of VSV as the membrane associated protein. Unlike retroviral envelope proteins, which bind to a specific cell surface protein receptor to gain entry into a cell, the VSV G protein interacts with a phospholipid component of the plasma membrane (Mastromarino *et al.*, J. Gen. Virol. 68:2359 [1977]). Because entry of VSV into a cell is not dependent upon the presence of specific protein receptors, VSV has an extremely broad host range. Pseudotyped retroviral vectors bearing the VSV G protein have an altered host range characteristic of VSV (*i.e.*, they can infect almost all species of vertebrate, invertebrate and insect cells). Importantly, VSV G-pseudotyped retroviral vectors can be concentrated 2000-fold or more by ultracentrifugation without significant loss of infectivity (Burns *et al.* Proc. Natl. Acad. Sci. USA 90:8033 [1993]).

The present invention is not limited to the use of the VSV G protein when a viral G protein is employed as the heterologous membrane-associated protein within a viral particle (*See, e.g.*, U.S. Pat. No. 5,512,421). The G proteins of viruses in the Vesiculovirus genera other than VSV, such as the Piry and Chandipura viruses, are highly homologous to the VSV G protein and, like the VSV G protein, contain covalently linked palmitic acid (Brun *et al.* Intervirol. 38:274 [1995] and Masters *et al.*, Virol. 171:285 (1990]). Thus, the G protein of the Piry and Chandipura viruses may be used in place of the VSV G protein for the pseudotyping of viral particles. In addition, the VSV G proteins of viruses

within the Lyssa virus genera such as Rabies and Mokola viruses show a high degree of conservation (amino acid sequence as well as functional conservation) with the VSV G proteins. For example, the Mokola virus G protein has been shown to function in a manner similar to the VSV G protein (*i.e.*, to mediate membrane fusion) and therefore may be used in place of the VSV G protein for the pseudotyping of viral particles (Mebatsion *et al.*, J. Virol. 69:1444 [1995]). Viral particles may be pseudotyped using either the Piry, Chandipura or Mokola G protein, with the exception that a plasmid containing sequences encoding either the Piry, Chandipura or Mokola G protein under the transcriptional control of a suitable promoter element (*e.g.*, the CMV intermediate-early promoter; numerous expression vectors containing the CMV IE promoter are available, such as the pcDNA3.1 vectors (Invitrogen)) is used in place of pHCMV-G. Sequences encoding other G proteins derived from other members of the Rhabdoviridae family may be used; sequences encoding numerous rhabdoviral G proteins are available from the GenBank database.

The majority of retroviruses may transfer or integrate a double-stranded linear form of the virus (the provirus) into the genome of the recipient cell only if the recipient cell is cycling (*i.e.*, dividing) at the time of infection. Retroviruses which have been shown to infect dividing cells exclusively, or more efficiently, include MLV, spleen necrosis virus, Rous sarcoma virus and human immunodeficiency virus (HIV; while HIV infects dividing cells more efficiently, HIV can infect non-dividing cells).

It has been demonstrated that the integration of MLV virus DNA depends upon the host cell's progression through mitosis and it has been postulated that the dependence upon mitosis reflects a requirement for the breakdown of the nuclear envelope in order for the viral integration complex to gain entry into the nucleus (Roe *et al.*, EMBO J. 12:2099 [1993]). However, as integration does not occur in cells arrested in metaphase, the breakdown of the nuclear envelope alone may not be sufficient to permit viral integration; there may be additional requirements such as the state of condensation of the genomic DNA (Roe *et al., supra*).

For example, in one such embodiment, the construct backbone may comprise: 1) the Murine Sarcoma Virus 5' LTR; 2) extended viral packaging signal (ψ); 3) a selectable marker (*e.g.*, neo); 4) an inducible promoter with an appropriate cloning site (*e.g.*, the native BLV U3 promoter); and 5) the 3' Murine Leukemia Virus 3' LTR. In some embodiments, a post-transcriptional enhancer element may be added to the retroviral vector backbone construct to optimize the transport of the message from the nucleus to the cytoplasm.

In some embodiments, the vectors may utilize the following items: 5' LTR (*e.g.*, MoMSV; extended packaging region; NEO; retroviral promoter (*e.g.*, BLV); gene of Interest; 3'LTR (*e.g.*, MoMuLV). Exemplary vectors of the present invention comprising YFP (yellow fluorescent protein) or M4 as an exemplary gene of interest are shown in **FIGS. 14-16** (SEQ ID NOs: 12 and 13). **FIG. 14** shows a map of a construct that further comprises a WPRE element.

The present invention also provides the use of lentiviral vectors to generate high copy number cell lines. The lentiviruses (*e.g.*, equine infectious anemia virus, caprine arthritis-encephalitis virus, human immunodeficiency virus) are a subfamily of retroviruses that are able to integrate into non-dividing cells. The lentiviral genome and the proviral DNA have the three genes found in all retroviruses: *gag, pol*, and *env*, which are flanked by two LTR sequences. The gag gene encodes the internal structural proteins (*e.g.*, matrix, capsid, and

nucleocapsid proteins); the pol gene encodes the reverse transcriptase, protease, and integrase proteins; and the pol gene encodes the viral envelope glycoproteins. The 5' and 3' LTRs control transcription and polyadenylation of the viral RNAs. Additional genes in the lentiviral genome include the *vif*, *vpr*, *tat*, *rev*, *vpu*, *nef*, and *vpx* genes.

A variety of lentiviral vectors and packaging cell lines are known in the art and find use in the present invention (*See, e.g.*, U.S. Pat. Nos. 5,994,136 and 6,013,516). Furthermore, the VSV G protein has also been used to pseudotype retroviral vectors based upon the human immunodeficiency virus (HIV) (Naldini *et al.*, Science 272:263 [1996]). Thus, the VSV G protein may be used to generate a variety of pseudotyped retroviral vectors and is not limited to vectors based on MoMLV. The lentiviral vectors may also be modified as described above to contain various regulatory sequences (*e.g.*, signal peptide sequences, RNA export elements, and IRES's). After the lentiviral vectors are produced, they may be used to transfect host cells as described above for retroviral vectors.

The present invention is not limited to the use of retroviral expression vectors. Any suitable expression vector for the expression of a gene of interest using the inducible promoters of the present invention may be utilized. Exemplary expression vectors include, but are not limited to cosmids, plasmids, adenoviral, and adeno-associated viral vectors. For example, in some embodiments, plasmids may be utilized for the expression of a gene of interest in a prokaryotic or eukaryotic cell. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Such vectors include, but are not limited to, the following vectors: 1) Bacterial -- pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); and 2) Eukaryotic -- pWLNEO, pSV2CAT, pOG44, PXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). Any other plasmid or vector may be used as long as it is replicable and viable in the host.

In some embodiments, mammalian expression vectors may comprise an origin of replication, a suitable promoter (*e.g.*, the inducible promoters of the present invention) and enhancer, and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. In other embodiments, DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

In certain embodiments, the DNA sequence in the expression vector may be operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. Inducible promoters useful in the present invention may include, but are not limited to, those described above. In other embodiments, recombinant expression vectors may include origins of replication and selectable markers permitting transformation of the host cell (*e.g.*, dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or tetracycline or ampicillin resistance in *E. coli*).

In some embodiments, transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Enhancers useful in the present invention include, but are not limited to, the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

In other embodiments, the expression vector may also contain a ribosome binding site for translation initiation and a transcription terminator. In still other embodiments, the vector may also include appropriate sequences for amplifying expression.

In still other embodiments, adenoviral and adeno-associated viral vectors may be utilized as expression vectors (See *e.g.*, WO 00/12738 and WO 00/09675 and U.S. Pat. Appl. Nos. 6,033,908, 6,019,978, 6,001,557, 5,994,132, 5,994,128, 5,994,106, 5,981,225, 5,885,808, 5,872,154, 5,830,730, and 5,824,544).

D. Host Cells

The present invention is not limited to a particular type of host cell. Numerous host cells that the vectors of the present invention are able to replicate and produce protein in may be adapted for use therewith. In preferred embodiments, eukaryotic host cells are utilized. In particularly preferred embodiments, mammalian host cells are utilized. A number of mammalian host cell lines are known in the art. In general, these host cells are capable of growth and survival when placed in either monolayer culture or in suspension culture in a medium containing the appropriate nutrients and growth factors, as is described in more detail below. Typically, the cells are capable of expressing and secreting large quantities of a particular protein of interest into the culture medium. Examples of suitable mammalian host cells include, but are not limited to, Chinese hamster ovary cells (CHO-K1, ATCC CCI-61); bovine mammary epithelial cells (ATCC CRL 10274; bovine mammary epithelial cells); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture; *see, e.g.*, Graham *et al.*, J. Gen Virol., 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor

(MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383:44-68 [1982]); MRC 5 cells; FS4 cells; rat fibroblasts (208F cells); MDBK cells (bovine kidney cells); and a human hepatoma line (Hep G2).

The inventors also contemplate the use of amphibian and insect host cell lines. Examples of suitable insect host cell lines include, but are not limited to, mosquito cell lines (*e.g.*, ATCC CRL-1660). Examples of suitable amphibian host cell lines include, but are not limited to, toad cell lines (*e.g.*, ATCC CCL-102).

II. Inducers

The expression vectors and systems of the present invention may utilize inducers (*e.g.*, transactivators). In preferred embodiments, the inducers are specific for the activation domain of the promoter chosen. For example, in some embodiments, the BLV promoter is activated with the BLV Tax inducer protein (SEQ ID NO:2). In other embodiments, the HTLV-1 promoter (SEQ ID NO:3) is activated by HTLV Tax protein (SEQ ID NO:4) and the HIV promoter (SEQ ID NO:5) is activated by the HIV Tat inducer protein (SEQ ID NO:6). In some embodiments, the HIV Tat protein is engineered to activate the BLV promoter (*e.g.*, via site directed mutagenesis; See *e.g.*, below discussion of engineered mutants).

The inducers of the present invention may be provided by any suitable method. In preferred embodiments, inducers are provided when expression of the gene of interest is desired (i.e., the expression of the gene of interest is regulated by the presence of inducer protein). In some embodiments, purified inducer protein is added to cells in culture. In other embodiments, a second expression vector encoding the inducer protein is included in the host cell comprising the retroviral expression vector of the present invention as known in the art.

A. Direct Administration

Accordingly, in some embodiments, purified inducer protein is provided directly to cells growing in culture. For example, in some embodiments, cells comprising a vector of the present invention are grown to the desired level of confluency for induction of protein expression and the inducer protein (*e.g.*, Tax) is added to the culture. In preferred embodiments, one or more additional agents or delivery techniques designed to aid in the ability of the inducer to cross the cell membrane are utilized. Exemplary administration techniques include, but are not limited to, electroporation, lipid encapsulation, and the fusion of a protein transduction sequence to the inducer protein.

Accordingly, in some embodiments, inducer proteins are administered via electroporation (See *e.g.*, Sambrook *et al.*, supra). In electroporation, a brief electrical charge is administered to a sample of cells in culture, briefly allowing proteins to pass through the cell membrane. Apparatuses for administering the charge are commercially available.

In other embodiments, inducer proteins are encapsulated in lipids to allow for their movement through cell membranes. Several commercially available reagents are available for use in lipid encapsulation (*e.g.*, including, but not limited to, Promega Corp., Madison, WI, Mirus Corp., Madison, WI; EquiBio, Middlesex, UK; Roche Applied Science, Indianapolis, IN; and Stratagene, La Jolla, CA).

B. Expression of an Inducer Gene

In other embodiments, inducer proteins are provided via a second vector in the cell of interest. In preferred embodiments, the second vector only expresses the inducer protein when it is desired to induce expression of the gene of interest. For example, in some embodiments, the vector comprising the inducer gene is introduced to the cell containing the expression vector comprising the gene of interest only when it is desired to induce expression of the gene of interest. In other embodiments, the second vector comprising the gene expressing the inducer gene is present at all times but is under the control of an inducible promoter. In still further embodiments, both a first vector comprising the gene of interest under control of an inducible retroviral vector and a second vector comprising an inducer gene under the control of a promoter (*e.g.*, a constitutive promoter) are introduced into a cell at the same time.

FIGS. 10-13 show exemplary vectors for the expression of Tax inducer protein (SEQ ID NOs: 10 and 11). **FIG. 12** shows an example of a vector further comprising a WPRE element. The present invention is not limited to the particular constructs shown in **FIGS. 10-13**. Additional vectors for the expression of inducer proteins may be utilized (See *e.g.*, above description of vectors).

C. Engineered Inducers

In some embodiments, inducer polypeptides (*e.g.*, Tax) are engineered to make them more able to pass through cell membranes. For example, in some embodiments, inducer proteins are generated as a fusion protein with an additional polypeptide sequence (*e.g.*, a protein transduction sequence) that allows them to pass through cell membranes unaided. In some embodiments, inducer proteins are fused to the HIV vp22 protein translocation sequence (See *e.g.*, U.S. Patents 6,358,739 and 6,251,398).

In other embodiments, inducer polypeptides such as Tax are generated as fusion proteins with the membrane translocation region of the HIV-1 Tat protein. The HIV Tat protein is known to cross cell membranes unaided (See *e.g.*, U.S. Patent 6,358,739; Vives *et al.*, J. Biol. Chem. 272:16010 [1997]; Futaki *et al.*, J. Biol. Chem. 276:5839 [2000]). The region of Tat known to direct translocation across cell membranes (i.e., the protein translocation domain) has been characterized (See *e.g.*, Vives *et al.*, supra; Futaki *et al.*, supra). Proteins fused to the protein translocation domain of Tat have been shown to cross cell membranes (See *e.g.*, Schwarze *et al.*, Science 285:1569 [1999]; Bhorade *et al.*, Bioconjug chem. 11:301 [2000]). Accordingly, in some embodiments, the protein translocation domain of Tat (*e.g.*, amino acids 48-60) is fused to an inducer protein of the present invention (*e.g.*, BLV or HTLV Tax).

In still further embodiments, an inducer protein of the present invention may be engineered to comprise an HIV Tat protein translocation domain internally. In preferred embodiments, the corresponding region of BLV or HTLV Tax (*e.g.*, as determined by protein homology searches) may be substituted with the protein translocation domain of Tat.

III. Kits

In some embodiments, the inducible gene expression system(s) of the present invention may be provided as a kit for the expression of a protein of interest. Such expression kits may include the essential reagents required for the inducible expression methods of the present invention. In some embodiments, the kit may include a vessel containing an expression vector (*e.g.*, a retroviral vector, plasmid, cosmid, adeno-associated viral vector, or an adenoviral vector) comprising an inducible promoter and a site for cloning an exogenous gene of interest such that the gene of interest is placed under the control of the inducible promoter. In some embodiments, the expression vector may further include an RNA export element (*e.g.*, a WPRE element).

In some embodiments, the kit may further include a purified inducer protein or source of inducer protein (*e.g.*, a vector for the expression of inducer protein) provided in a separate vessel. In the case of kits comprising a second expression vector for expression of the inducer protein, the kit may include all the reagents necessary for transfer of the second expression vector into a host cell and expression of the inducer protein.

The concentration of inducer protein may vary depending on the inducer protein and promoter, which may be determined experimentally. The kits according to the present invention may comprise a vessel containing excess amounts of inducer protein, such that the level can be optimized by the end user. The inducer protein provided in the kit may be at a high enough concentration such that only a small volume of inducer is required for inducing expression from the promoters.

In some embodiments, the kits may further include a separate vessel comprising host cells for expression of the gene of interest. The exact type of host cell, concentration of host cells, and storage conditions, are dependent on the type of expression vector chosen (*e.g.*, plasmid or viral vector; eukaryotic or prokaryotic host cells).

The reagents may be provided in containers and be of a concentration suitable for direct use or use after dilution. A control may also be provided to allow for control of gene expression results. The kit may further include buffer for performing cloning and/or expression of the inducer protein. The kit may be packaged in a single enclosure including instructions for performing the expression methods. The instructions provided with the kit are specific to the type of expression vector and host cell provided with the kit. For example, separate kits may be provided for either viral or plasmid expression vectors.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: M (molar); mM (millimolar); µM (micromolar); nM (nanomolar); mol (moles); mmol (millimoles); µmol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams); µg (micrograms); pg (picograms); L (liters); ml (milliliters); µl (microliters); cm (centimeters); mm (millimeters); µm (micrometers); nm (nanometers); and °C (degrees Centigrade).

Example 1:

Tax Inducible Gene Expression

Two different promoter constructs were analyzed in this example. **Table 1** shows the constructs utilized in the experiments below and the geometric mean brightness values from FACS analysis. The BLV promoter was compared to the immediate early promoter of cytomegalovirus (CMV), a known strong promoter for mammalian expression systems. Two reporter constructs were used, LNBlv-YFP (SEQ ID NO:13), which contains the gene for yellow fluorescent protein (YFP) under the control of the BLV promoter, and LNC-YFP, which contains the gene for YFP under the control of the CMV promoter. The reporter constructs utilize the Neo selectable marker. Two inducer constructs were used, LBC-BTax (SEQ ID NO:10), which contains the gene for BLV Tax under control of the CMV promoter, and LBC-BTaxW, which is the same as LBC-BTax, with the addition of the WPRE in the 3' UTR of the BTax gene. The addition of the WPRE sequence to the Tax message is contemplated to increase Tax protein expression and lead to higher induction of the BLV promoter. D17 (canine osteogenic sarcoma) cells were then transduced with these vectors in the combinations shown in **Table 1**.

Promoter strength was qualitatively and quantitatively evaluated by fluorescence activated cell sorting (FACS) and Western blot analysis, respectively, measuring yellow fluorescent protein (YFP) expression in cells transfected with the various vectors. The amount of YFP expression observed was directly proportional to promoter strength and, therefore, gene expression levels.

Table 1.

Cell Line (All D17)	**Transduced With Retroviral Vector(s)**	**Geometric Mean Brightness (FACS)**		
(-)	None (negative control)	1.17		
BY	LNBlv-YFP	29.55		
CY	LNC-YFP	441.52		
		Unsorted	Bright	Dim
BY + Tax	LNBlv-YFP + LBC-BTax	324.18	545.79	31.55
BY+TaxW	LNBlv-YFP + LBC-BTaxW	59.65	2310.02	29.93
CY + Tax	LNC-YFP + LBC-BTax	492.65		
CY+TaxW	LNC-YFP + LBC-BTaxW	678.83		

For the FACS analysis, propidium iodide (PI) was first added to the cells as an indicator of dead cells. The cells were gated according to PI signal such that only live cells were analyzed. Non-transfected D17 cells were used as a control for no YFP expression. The YFP expression of BY, CY, BY+Tax, CY+Tax, BY+TaxW, and CY+TaxW cells was measured and compared to non-transduced D17 cells [(-) in **Table 1**]. For each sample, YFP expression was quantified as amplitude of YFP. The YFP amplitude was on a logarithmic scale; therefore, YFP expression was measured as a geometric mean. The geometric mean data were organized into histograms that showed the distribution of YFP expression across a population of 10,000 cells. For the BY+Tax and BY+TaxW cells, the cell sorter was used to characterize the cells into Bright and Dim populations, as well as the whole population (Unsorted). With TaxW, a majority of the cells showed weak YFP expression, however about 20% of the cells showed extreme brightness.

FACS analysis was used to investigate the differences in YFP expression and, therefore, gene expression between the CMV and the BLV promoter with and without the Tax inducer. In the absence of Tax, the CMV promoter gave good expression of YFP, which was similar when the Tax or TaxW inducer protein was co-expressed (**FIG. 1**, **Table 1**). For the BLV promoter, YFP expression in the absence of Tax or TaxW was weak (BY **FIG. 1** and **Table 1**) – barely detectable by FACS sorting. Adding the Tax inducer protein (in the absence of WPRE, "BY + Tax" in **FIG. 1** and **Table 1**), increased YFP expression to levels comparable to YFP expression driven from the CMV promoter. When the WPRE was added to the Tax message, presumably increasing levels of Tax inducer protein, a subpopulation of cells showed extremely bright fluorescence. The Green Fluorescent Protein and its derivatives (YFP) are known to be toxic to cells when expressed at high levels. Thus, it is contemplated that very high expression led to selection of cells with low expression (the majority of cells – Dim) with a subpopulation expressing at full induction of the BLV promoter

(Bright cells). In this case, the BY+TaxW cells (bright) showed levels of YFP expression 4-5 fold higher than CMV promoter driven YFP cells as judged by geometric mean fluorescence (**FIG. 1**, **Table 1**).

Quantitative western blot analysis was also performed on the six transfected cell types. The cells were first lysed using the M-PER (Pierce Biotechnology, Rockford, IL) mammalian protein extraction reagent. Cell lysates were then loaded onto a 12.5% SDS polyacrylamide gel electrophoresis system along with YPF sample standards of various concentrations. Following separation, the gel was transferred to a nitrocellulose membrane using the CRITERION gel blotting system (BioRad, Hercules, CA). The nitrocellulose membrane with the proteins attached was then treated in a blocking solution, followed by the primary murine anti-YFP specific and secondary dye-labeled rabbit anti-murine antibodies, and finally treatment with chemiluminescent reagent. The YFP treated with the chemiluminescent reagent was then exposed to x-ray film and developed. The bands produced on the film corresponded to YFP expression. Using the Un-Scan-It software program (available at the internet web site of ScienceDownload.com), the amount of YFP expression was quantitatively measured in terms of pixels on the film. Using the YFP standards, a standard curve was produced correlating the mass of YFP in the sample with pixels enumerated. From the standard curve, the mass of YFP in the BY, CY, BY+Tax, CY+Tax, BY+TaxW, and CY+TaxW samples was extrapolated. As with the FACS data, BLV promoter driven expression of YFP was very low in the

absence of the Tax inducer. The co-expression of Tax increased BLV promoter driven YFP expression to a level similar to YFP expression from the CMV promoter.

Table 2. Quantitative Western

	YFP Expression ngs +/- (StDev)
BY	not detected
BY+Tax	43.1 (7.4)
BY+TaxW	42.7 (0.8)
CY	79.2 (12.0)
CY+Tax	66.9 (21.5)
CY+TaxW	76.3 (18.7)

Example 2:

Comparison of BLV and CMV Promoter-Driven Reporter Gene Expression in BLV-Infected and Non-Infected Cells

A commercially available retroviral system was used with its standard CMV promoter (CMVp) or replaced with the BLV promoter (BLVp). **FIG. 17** shows a schematic of the BLV promoter used in this experiment with its unique regulatory elements. The luciferase reporter gene was used to compare promoter expression strength within different cell lines and treatments. The WPRE was also incorporated to enhance transgene expression within these retroviral vectors. WPRE has been reported to significantly stimulate expression of transgenes in a promoter-independent fashion. Retroviral vectors were used because of the ease of stable cell line establishment, and because of its

prominent use in transgenics and gene therapy. Cells of several different tissues and species were used in our studies and are listed in **Table 3**.

Table 3. Cells Used in Promoter Comparison Studies

Name	Description	Reference
D17	Dog Osteosarcoma	ATCC CCL-183; (Boris-Lawrie *et al.*, J. Virol. 71:1514 [1997])
FLK	Sheep Kidney Cell Line; BLV expresser	(Tajima *et al.*, J. Virol. 77:1984 [2003])
BL3.1	Cow B-Lymphosarcoma; BLV expresser	ATCC CRL-2306; (Harms *et al.*, Hum. Gene Ther. 6:1291 [1996])
B-Cell	Primary Cow B cell	Isolated as previously described (Harms *et al.*, Hum. Immunol. 44:50 [1995]) washed, and magnetically separated as known in the art.

It was established through experimentation that the BLV promoter may be as strong as the CMV promoter depending on the host cell. Mammalian expression vector promoters have complex *cis* elements that bind diverse cellular *trans* factors resulting in varied levels of *trans*-gene expression depending on the host cell. Although the CMV promoter induces high-level constitutive *trans*-gene expression in most cells, absolute levels of expression vary greatly from cell line to cell line. In several cell lines (Harms *et al.*, Hum. Immunopathology. 51:39 [1996]), the D17 cell line may produce the greatest level of *trans*-gene product through the CMV promoter. In contrast to the constitutive expression of the CMV promoter, the BLV promoter has *cis* elements that are dependent on BLV Tax for

transgene expression (Tajima *et al.*, J. Virol. 77:1984 [2003]). It was hypothesized therefore that in a cell line such as D17, the BLV promoter would have little or no activity compared to the CMV promoter. Conversely, in a cell line expressing the BLV Tax transactivator such as the BLV-producing FLK cell line, the BLV promoter would have similar activity compared to the CMV promoter. This theory was tested with luciferase as the transgene.

It was determined that the BLV promoter activity was about 50-fold less than CMV promoter activity in D17 cells but was about equal in FLK cells (**FIG. 18A**). As shown in **FIG. 1**, the BLVp also has a *cis* element that is B cell specific (PU.1/Spi-B). The strengths of BLV and CMV promoters were compared in primary B cells and a BLV infected B cell line hypothesizing that BLVp expression would be comparable to CMVp activity. BLVp activity was still less than CMVp activity in primary B cells but by only about a 5-fold difference (**FIG. 18B**). In the BLV infected BL3.1 cell line, BLVp activity was about equal to CMVp activity, analogous to results using the BLV infected FLK cell line. Thus, the BLV promoter may be as strong as the CMV promoter under appropriate conditions (e.g., BLV infection/Tax expression).

Example 3:

BLV infection enhances BLV promoter expression but has no measurable effect on the CMV promoter

BLV promoter activity was greater than CMV promoter activity in the BLV infected FLK cell line but minimal compared to CMV promoter activity in the non-BLV infected D17 cell line. A determination was made as to whether BLV infection of D17 cells would enhance BLVp and/or suppress CMVp expression. The dog derived D17 cell line can be infected with BLV albeit not very efficiently (Boris-Lawrie *et al.*, J. Virol. 71:1514 [1997]). D17 cells were infected with concentrated BLV from FLK cells, then clonally selected for BLV expression using pol RT-PCR and BLV reverse transcriptase assay of the supernatant. Luciferase assays demonstrated that BLV promoter activity in infected D17 cells was about 10-fold greater than BLV promoter activity in non-infected D17 cells (**FIG. 19**). CMV promoter activity remained unchanged.

The CMV promoter activity was still about 5-fold greater than BLV promoter activity in the BLV infected D17 cells compared to the relatively equal activity of the CMV promoter versus BLV promoter in BLV infected FLK cells. However, FLK cells are thought to contain four copies of the BLV provirus whereas BLV infected D17 cells contain a single copy of the provirus. Thus, there may be relatively greater expression of Tax in FLK cells effecting greater activity of the BLV promoter. Quantitative levels of Tax in BLV infected D17 or FLK cells were not measured.

Example 4:

BLV Tax enhances BLV promoter expression but has no measurable effect on the CMV promoter

The BLV provirus encodes Tax within the X region located between the env gene and the 3' long terminal repeat (LTR). Within the provirus, Tax is subject to complex transcriptional and posttranscriptional regulation necessary for BLV expression and infectivity (Van Den Broeke *et al.*, J. Virol. 731:1054 [1999]). Non-Tax regulatory factors encoded by BLV may affect the host and directly or indirectly, BLV promoter expression (Kerkhofs *et al.*, J. Virol. 72:2554[1998]). To assess directly the effect of constitutive Tax expression on the BLV promoter and CMV promoter, BLV Tax was provided as a trans-gene to cells. As expected, Tax significantly enhanced BLV promoter activity but had no measurable effect on CMV promoter activity (**FIGS. 20A/B**) inducing BLVp activity about 48-fold in D17 cells. When BLV infected cells were transduced with the Tax transgene, the resulting increase in BLV promoter activity was a

greater-than-additive enhancement of BLV infection and Tax transgene (**Table 4**). This effect could likely be caused by Tax up-regulating its expression within the BLV provirus. The effect on the CMV promoter was not significant.

Table 4. Percent of Basal Luciferase Expression

Promoter	D17+Tax	D17+BLV	D17+Tax+BLV
BLVp	115±7	1226±15	2038±202
CMVp	96±5	130±23	118±14

It should be noted that throughout the aforementioned studies of Examples 2, 3, and 4, expression vectors were assayed with and without the WPRE. WPRE enhanced transgene expression in all cell lines used, and in a promoter-independent fashion (about 2-fold greater for BLVp and CMVp in D17 cells). All data provided relating to Examples 2, 3, and 4 include vectors containing WPRE.

Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in material science, chemistry, and molecular biology or related fields are intended to be within the scope of the following claims.